

SELENIUM HYPERACCUMULATION BY *ASTRAGALUS* (FABACEAE) DOES NOT INHIBIT ROOT NODULE SYMBIOSIS¹

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- *Premise of study:* A survey of the root-nodule symbiosis in *Astragalus* and its interaction with selenium (Se) has not been conducted before. Such studies can provide insight into how edaphic conditions modify symbiotic interactions and influence partner coevolution. In this paper plant-organ Se concentration ([Se]) was investigated to assess potential Se exposure to endophytes.
- *Methods:* Selenium distribution and molecular speciation of root nodules from Se-hyperaccumulators *Astragalus bisulcatus*, *A. praelongus*, and *A. racemosus* was determined by Se K-edge x-ray absorption spectroscopy. A series of greenhouse experiments were conducted to characterize the response of root-nodule symbiosis in Se-hyperaccumulators and nonhyperaccumulators.
- *Key results:* Nodules in three Se-hyperaccumulators (*Astragalus crotalariae*, *A. praelongus*, and *A. preussii*) are reported for the first time. Leaves, flowers, and fruits from Se-hyperaccumulators were routinely above the hyperaccumulator threshold (1,000 $\mu\text{g Se g}^{-1}$ DW), but root samples rarely contained that amount, and nodules never exceeded 110 $\mu\text{g Se g}^{-1}$ DW. Nodules from *A. bisulcatus*, *A. praelongus*, and *A. racemosus* had Se throughout, with a majority stored in C-Se-C form. Finally, an evaluation of nodulation in Se-hyperaccumulators and nonhyperaccumulators indicated that there was no nodulation inhibition because of plant Se tolerance. Rather, we found that in Se-hyperaccumulators higher levels of Se treatment (up to 100 $\mu\text{M Se}$) corresponded with higher nodule counts, indicating a potential role for dinitrogen fixation in Se-hyperaccumulation. The effect was not found in nonhyperaccumulators.
- *Conclusions:* As the evolution of Se hyperaccumulation in *Astragalus* developed, root-nodule symbiosis may have played an integral role.

Key words: adaptation; legume; plant-microbe; selenium

Several hyperaccumulator taxa and metallophytes are members of the legume family (Fabaceae). Many of these species are within the Papilionoideae subfamily, which has been reported to have more than 98% of its members form root nodules (Allen and Allen, 1981). Therefore, despite the challenges of being rooted in metalliferous soils, leguminous hyperaccumulators may be expected to form root-nodule symbioses. However, to date our knowledge of whether and how leguminous Se-hyperaccumulators interact with symbiotic rhizobia is limited. For metal-tolerant symbiotic legumes to evolve, tolerance needs to occur in both the plant and the bacterial partner

(Antonovics et al., 1971). Symbiotic bacteria within root nodules are enclosed in the peribacterioid membrane and are exposed to consistent environmental conditions within the plant cell and may have some protection from stress, unlike free-living rhizobia that are more susceptible to stress and environmental fluctuations in the soil environment and rhizosphere (Chalk et al., 2010).

The *Astragalus* genus makes a good model system to evaluate how hyperaccumulation affects nodulation characteristics. The vast majority of *Astragalus* species do not hyperaccumulate elements, but a select number of species native to western North America do hyperaccumulate Se. The species that hyperaccumulate Se manage to amass large concentrations, while co-occurring congeners do not accumulate Se to any large extent (Shrift 1969; Galeas et al., 2007). Investigating *Astragalus* species may indicate if there is a coevolutionary relationship between plant hyperaccumulation and root-nodule microorganisms. Three alternative pathways could have developed: (i) plants that have evolved to hyperaccumulate Se may associate with rhizobia that have evolved to interact with high [Se] within hyperaccumulator plants and rhizospheres; (ii) the presence of Se in the system could disrupt the symbiosis entirely where Se-hyperaccumulators rarely nodulate or form ineffective partnerships; or (iii) there could be no change in interaction in response to Se.

Under the first alternative, symbiotic interactions may enhance host stress tolerance. Some plants have been shown to require

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TABLE 1. Collection locations of the plants used to assess organ [Se] in the field including the closest Colorado town and soil Se type, plant species and hyperaccumulation type, UTM coordinates (NAD83/WGS84), and site elevation (m).

Site and Species	Type	UTM Zone	Easting	Northing	Elevation (m)
Fort Collins	Seleniferous site	13N	490631	4506616	1597
<i>Astragalus bisulcatus</i>	Hyperaccumulator				
<i>Astragalus missouriensis</i>	Nonhyperaccumulator				
<i>Melilotus officinalis</i>	Nonhyperaccumulator				
<i>Oxytropis sericea</i>	Nonhyperaccumulator				
Meeker	Nonseleniferous site	12N	722225	4383948	2466
<i>Astragalus argophyllus</i>	Nonhyperaccumulator				
<i>Astragalus convallarius</i>	Nonhyperaccumulator				
Pueblo	Seleniferous site	13N	539143	4242337	1536
<i>Astragalus racemosus</i>	Hyperaccumulator				
<i>Melilotus albus</i>	Nonhyperaccumulator				
Uravan	Seleniferous site	12N	697009	4249798	1504
<i>Astragalus praelongus</i>	Hyperaccumulator				
<i>Astragalus rafaensis</i>	Hyperaccumulator				

symbiotic associations under certain conditions of environmental stress that are not limited to low nutrient availability. As an example, thermal tolerance and salt tolerance were only achieved in plants growing in symbiosis with mycorrhizae (Rodriguez et al., 2008). Yet, under the second alternative described above, stress may disrupt symbiotic relationships. An example of the disruptive influence of abiotic stress has been shown in the legume genus *Acacia* where more salt-tolerant plant species had less of a growth increase in response to rhizobial inoculation than did salt-sensitive host species; therefore plant salt-tolerance results in reduced dependence on the symbiotic mutualism (Thrall et al., 2008). In addition, cases of metal inhibition of nodulation and nitrogen fixation have been shown in other legumes (Chen et al., 2003; Manier et al., 2009; Saraswat and Rai, 2011). This type of alternative has previously been described with hyperaccumulator plants where a recent analysis of symbiotic interactions in Ni-hyperaccumulators indicated that the strongest Ni-hyperaccumulators were the least infected by mycorrhizae (Amir et al., 2007). To illustrate the third alternative, some metal-tolerant legumes have effective symbioses under stress conditions, as was observed for *Lotus purshianus* Clem. & E.G. Clem. and its symbiont growing in copper-mine waste (Wu and Kruckeberg, 1985; Wu and Lin, 1990). We sought to determine which pattern occurs in other symbiotic relationships established with hyperaccumulator plants.

An investigation of the literature provided clues from previous studies as to what may occur in *Astragalus* and their microsymbionts. In the case of Se-hyperaccumulation, it is known that some rhizobia isolates are tolerant to 200 mM Se(IV) and 400 mM Se(VI) in vitro (Kinkle et al., 1994). However these strains are not known to form root nodules with *Astragalus* species. Successful root-nodule symbioses exist in both *Astragalus* hyperaccumulators and non-accumulators (Allen and Allen, 1981).

From previous reports it is known that the Se-hyperaccumulators *A. bisulcatus* (Hook.) A. Gray, *A. pectinatus* (Douglas ex Hook.) Douglas ex G. Don, and *A. racemosus* Pursh are nodulated (Wilson and Chin, 1947; Lindblom et al., 2012), but no one knows how the symbiotic associations respond to Se. In *A. bisulcatus*, root [Se] can reach levels that are used to define plants as Se-hyperaccumulators ($>1,000 \mu\text{g Se g}^{-1}$ DW shoot) (Galeas et al., 2007; Barillas et al., 2011). With roots having high [Se], nodules of hyperaccumulators potentially experience similar [Se]. We wanted to compare the effect of Se in nodulation of *Astragalus* species that have evolved to hyperaccumulate Se to those that have not. In this study, we address the question—does plant Se-hyperaccumulation affect root-nodule symbiosis?

We checked for the presence of nodulation and investigated the [Se] in organs of several *Astragalus* hyperaccumulators and leguminous nonhyperaccumulators. We hypothesized that Se-hyperaccumulation inhibited the symbiotic interaction, where hyperaccumulators have lost some of their ability to effectively interact with rhizobia. We expected nodulation in Se-hyperaccumulators and nonhyperaccumulators to be reduced with Se addition. To address this hypothesis we conducted field surveys and examined differences in nodulation between Se-hyperaccumulators and nonhyperaccumulators in greenhouse studies. Our studies also included Se localization and speciation analyses within root nodules from the three Se-hyperaccumulators *A. bisulcatus*, *A. praelongus*, and *A. racemosus* to determine how bacteria in nodules are subjected to Se within these plants.

MATERIALS AND METHODS

Nodulation occurrence in Se-hyperaccumulators—Seeds from *Astragalus crotolariae* (Benth.) A. Gray were obtained from the Desert Legume Program

TABLE 2. Collection locations for soil from beneath six *Astragalus* species used as inoculum in the experiment examining the effects of Se on nodulation including the closest Colorado town, UTM coordinates (NAD83/WGS84), and site elevation (m).

Species	Type	Location	UTM Zone	Easting	Northing	Elevation (m)
<i>A. bisulcatus</i>	Hyperaccumulator	Fort Collins	13N	490631	4506616	1597
<i>A. convallarius</i>	Nonhyperaccumulator	Meeker	12N	722225	4383948	2466
<i>A. drummondii</i>	Nonhyperaccumulator	Livermore	13N	470315	4512908	1946
<i>A. praelongus</i>	Hyperaccumulator	Uravan	12N	697009	4249798	1504
<i>A. racemosus</i>	Hyperaccumulator	Limon	13N	598433	4356258	1749
<i>A. shortianus</i>	Nonhyperaccumulator	Livermore	13N	470360	4512932	1940

(Tucson, Arizona, USA) and the USDA Western Regional Plant Introduction Station (Pullman, Washington, USA) provided seeds from *A. preussii* A. Gray. After the seed coats were scarified with a razor incision, the seeds were soaked overnight in tap water, and then sown into soil collected from underneath *A. bisulcatus* growing near Fort Collins, Colorado, USA. After 2 mo of growth 50 μM

sodium selenate solution (20 mL) was applied weekly for the remaining 4 mo, after which the plants were removed from their pots and examined for nodules. Root systems of *A. bisulcatus* (Fort Collins, Colorado), *A. praelongus* (Uravan, Colorado) and *A. racemosus* (Pueblo, Colorado) growing in their native habitats were also excavated to search for root nodules under natural conditions.

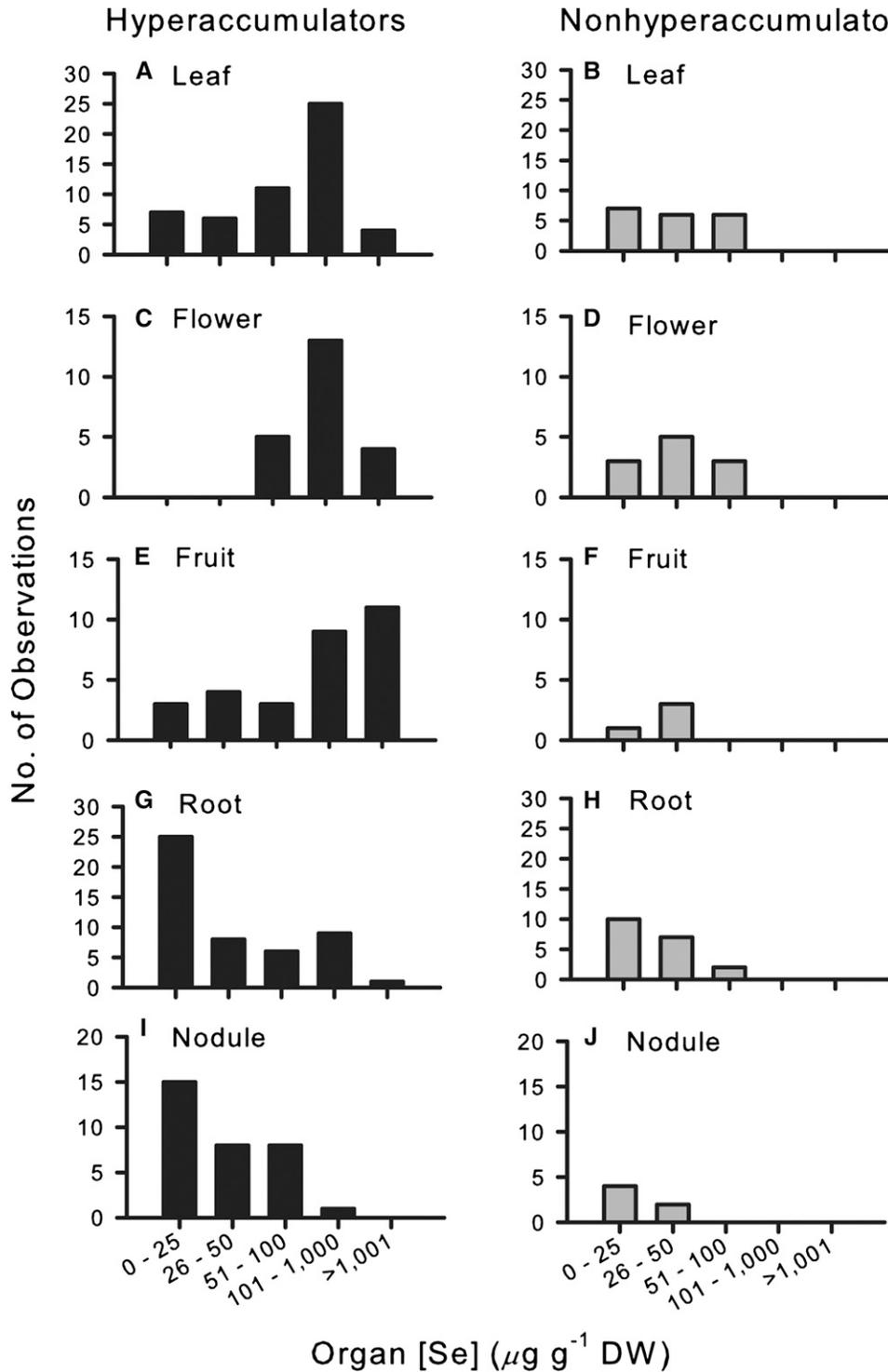


Fig. 1. Histograms of organ [Se] in four Se-hyperaccumulator species (*Astragalus bisulcatus*, *A. praelongus*, *A. racemosus*, and *A. rafaensis*) as shown for (A) leaf, (C) flower, (E) fruit, (G) root, and (I) nodule. The organ [Se] in six nonhyperaccumulator legumes (*A. argophyllus*, *A. convallarius*, *A. missouriensis*, *Melilotus albus*, *M. officinalis*, and *Oxytropis sericea*) are also shown for (B) leaf, (D) flower, (F) fruit, (H) root, and (J) nodule.

Organ [Se] from field collections—To assess potential Se exposure for microbial endophytes we determined plant organ [Se] from excavated root systems of several nonhyperaccumulators and several Se-hyperaccumulators during the growing season (Table 1). Aboveground organs including leaves, flowers, and fruits were separated from the belowground organs in the field. Within one day of collection the samples were returned to the laboratory and the belowground organs were washed and separated into roots and nodules. All parts were dried at 40°C, weighed, and ground. For inductively coupled plasma atomic emission spectrometry (ICP-AES), 20 mg of dried plant samples were digested in 1 mL nitric acid for 2 hours at 60°C and then 130°C for 6 hours (Zarcinas et al., 1987). Organ [Se] was determined by ICP-AES on this acid digest after a 10-fold dilution with water (Fassel, 1978). An 8-element standard mix was run as a quality control every 15 samples, if the standards failed (outside of +/- 20% acceptance margin) we restandardized and reran the samples. We used 1,000 ppm elemental standards from SPEX CertiPrep, Inc. (Metuchen, New Jersey, USA), diluted to 1 to 2 ppm.

Se localization and speciation in nodules—Root nodules of hyperaccumulators were obtained from *Astragalus bisulcatus*, growing in the field, along with *A. praelongus* Sheldon and *A. racemosus*, growing under greenhouse conditions exposed to 50 µM sodium selenate (as described above under Nodulation occurrence in Se-hyperaccumulators). Nodules were separated from the majority of the roots, washed in water to remove external Se, frozen in liquid N₂, and then sliced in half. The samples were kept frozen until analysis. Nodule Se localization and speciation were determined using microfocused x-ray fluorescence (µXRF) mapping and x-ray absorption near-edge structure (µXANES) spectroscopy at the Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Laboratory (Marcus et al., 2004). Frozen nodule samples were placed onto a Peltier stage kept at -27°C to minimize beam radiation damage. µXRF elemental distribution maps were recorded at 13 keV, using a 15 µm (H) × 6 µm (V) beam, 15 µm × 15 µm pixel size, and 50 ms dwell time per pixel. A seven-element germanium (Ge) solid-state detector (Canberra Industries, Concord, Ontario, Canada) was used to record µXRF maps and µXANES spectra. The chemical forms of Se in three spots per sample were investigated using Se K-edge XANES. Standard procedures were used to correct the spectra for deadtime, pre-edge background, and postedge normalization (Kelly et al., 2008). Spectra were calibrated with red Se (white-line position set at 12660 eV) and least-square linear combination (LSQ) fitted in the 12630–12850 eV range using a library of well-characterized Se standard compounds to identify chemical species (Freeman et al., 2006). The best fit was obtained for minimum normalized sum-squares residuals: $NSS = 100 \times \{ \sum (\mu_{exp} - \mu_{fit})^2 / \sum (\mu_{exp})^2 \}$, where μ represents the normalized absorbance. The error on the percentages of species present is estimated to be ± 10%. Se standards included: Na₂SeO₄, Na₂SeO₃, SeCystine, and SeMet purchased from Sigma-Aldrich (St. Louis, Missouri, USA), and MeSeCys, γGMeSeCys, SeCysth, and SeGSH₂ purchased from PharmaSe (Austin, Texas, USA). SeCys was obtained by reducing SeCystine at 25°C overnight in 100 mM sodium borohydride at a 1:1 molar ratio. Samples of gray and red elemental Se were provided by Amy Ryser and Dan Strawn (University of Idaho, Moscow, ID, USA). A suite of custom LabVIEW (National Instruments, Rockville, Maryland, USA) programs available at the beamline were used for data processing and analyses.

Root and nodule [Se] under greenhouse and field conditions—*Astragalus bisulcatus*, purchased from Western Native Seed (Coaldale, Colorado, USA), was grown from seed in 164-mL Ray Leach "Cone-tainers" (Stuewe & Sons,

Tangent, Oregon, USA) containing a 1:1 (v:v) mixture of sand and fritted clay at the University Greenhouse at Colorado State University. Seeds were scarified with sand-paper, sterilized with bleach for 2 min followed by 2 min in 90% ethanol, and soaked overnight in sterile water before planting in pots. The seedlings were allowed to establish for 1 mo before fertilizer treatments were applied. Fertilizer solution (1.6 mM K₂SO₄, 0.358 mM NH₄NO₃, 17.9 µM FeCl₃, 16.9 µM Na₂-EDTA, 2 mM MgSO₄, 0.169 mM KH₂PO₄, 0.833 mM K₂HPO₄, 0.6 mM CaSO₄, 0.5 mM CaCl₂, 23.1 µM H₃BO₃, 4.6 µM MnSO₄, 0.8 µM ZnSO₄, 0.3 µM CuSO₄, 0.2 µM Na₂MoO₄, and 0.5 µM CoSO₄) based on the Huss-Danell (1978) formulation was applied twice a week. The plants grew for 2 mo with fertilizer additions, then fertilizer application was suspended for 2 wk before inoculation and Se treatments were applied.

A soil sample was collected from *Astragalus bisulcatus* plants growing in a grassland community in Fort Collins, Colorado to make a soil slurry for inoculation (20 g soil in 1 L sterile, distilled water). The inoculant (20 mL) was added to pots directly. Six days after inoculation the fertilizer treatments were resumed with 1/4-strength fertilizer twice per week. After another 6 d, Se treatments (20 µM Na₂SeO₄) were started. Sodium selenate solutions were mixed in distilled water and supplied as 50-mL applications once every week. One month after Se treatments began the plants began to brown so fertilizer was reduced to once a week and Se treatments were reduced to once every 2 wk for the duration of the experiment (10 more weeks). Plants were harvested and separated into roots, shoots, and nodules. Each sample was dried at 50°C for one week before analysis.

Root and nodule [Se] was determined in samples from the greenhouse experiment as well as samples from *Astragalus bisulcatus*, *A. praelongus*, and *A. racemosus* collected from the field (as described under Organ [Se] from Field Collections). As previously described, ICP-AES was used to determine organ [Se] on digested samples. For statistical analysis we compared root and nodule [Se] in each species by *t*-test in Systat v.12 (Systat Software, Chicago, Illinois, USA).

Se effect on nodulation—We grew *Astragalus bisulcatus*, *A. convallarius* Greene, *A. drummondii* Douglas ex Hook., *A. praelongus*, and *A. shortianus* Nutt. obtained from Western Native Seed (Coaldale, Colorado, USA), and *A. racemosus* from Prairie Moon Nursery (Winona, Minnesota, USA) in a mixture of 2:1 (v:v) washed sand and field soil (sieved through a 2 mm mesh, Table 2). Soil collected from Se-hyperaccumulators and nonhyperaccumulators were similar in total (1.21 – 2.02 µg g⁻¹) and extractable (0.71 to 0.90 µg g⁻¹) Se (Sparks et al., 1996). The plants received 0, 50, or 100 µM sodium selenate (Na₂SeO₄) and an N-free fertilizer solution (0.4 mM K₂SO₄, 4.475 µM FeCl₃, 4.225 µM Na₂-EDTA, 0.5 mM MgSO₄, 0.042 mM KH₂PO₄, 0.208 mM K₂HPO₄, 5.775 µM H₃BO₃, 1.15 µM MnSO₄, 0.2 µM ZnSO₄, 0.075 µM CuSO₄, 0.05 µM NaMoO₄, and 0.125 µM CoSO₄) weekly, starting at 4 mo of age. After 2 mo of treatment, shoots were harvested and dried at 50°C while roots with nodules were immediately frozen at -20°C until subsequent harvesting could be conducted. After freezing, roots were harvested by washing to remove soil and separating nodules. Nodules were counted at harvest, kept at 40°C until completely dry, and then weighed to determine dry weight.

A comparison of the nodulation index (equivalent to number of nodules per gram shoot) where hyperaccumulators were expected to have a lower value than nonhyperaccumulators, consistent with symbiotic inhibition from Se, was conducted using a one-way *t*-test. The nodulation index was used to control for differences in size among species. In addition, a comparison of nodule size based on the average dry weight of each nodule in hyperaccumulators and nonhyperaccumulators was conducted using a one-way *t*-test where hyperaccumulators were

TABLE 3. Maximum [Se] (µg Se g⁻¹ DW) in different legume organs collected from the field for a survey of organ [Se] in Se-hyperaccumulators and nonhyperaccumulators. Some organs were not collected and elemental concentrations were not determined (–).

Plant type	Species	Leaf	Flower	Fruit	Root	Nodule
Nonhyperaccumulator	<i>Astragalus argophyllus</i>	47	58	–	66	0
	<i>Astragalus convallarius</i>	77	–	–	37	–
	<i>Astragalus missouriensis</i>	83	52	48	48	–
	<i>Melilotus albus</i>	39	40	28	17	26
	<i>Melilotus officinalis</i>	61	–	–	56	–
	<i>Oxytropis sericea</i>	12	20	5	25	29
Hyperaccumulator	<i>Astragalus bisulcatus</i>	436	606	291	65	77
	<i>Astragalus praelongus</i>	2,925	2,999	5,405	1,281	91
	<i>Astragalus racemosus</i>	422	81	83	87	109
	<i>Astragalus rafaellensis</i>	689	2,151	1,263	–	–

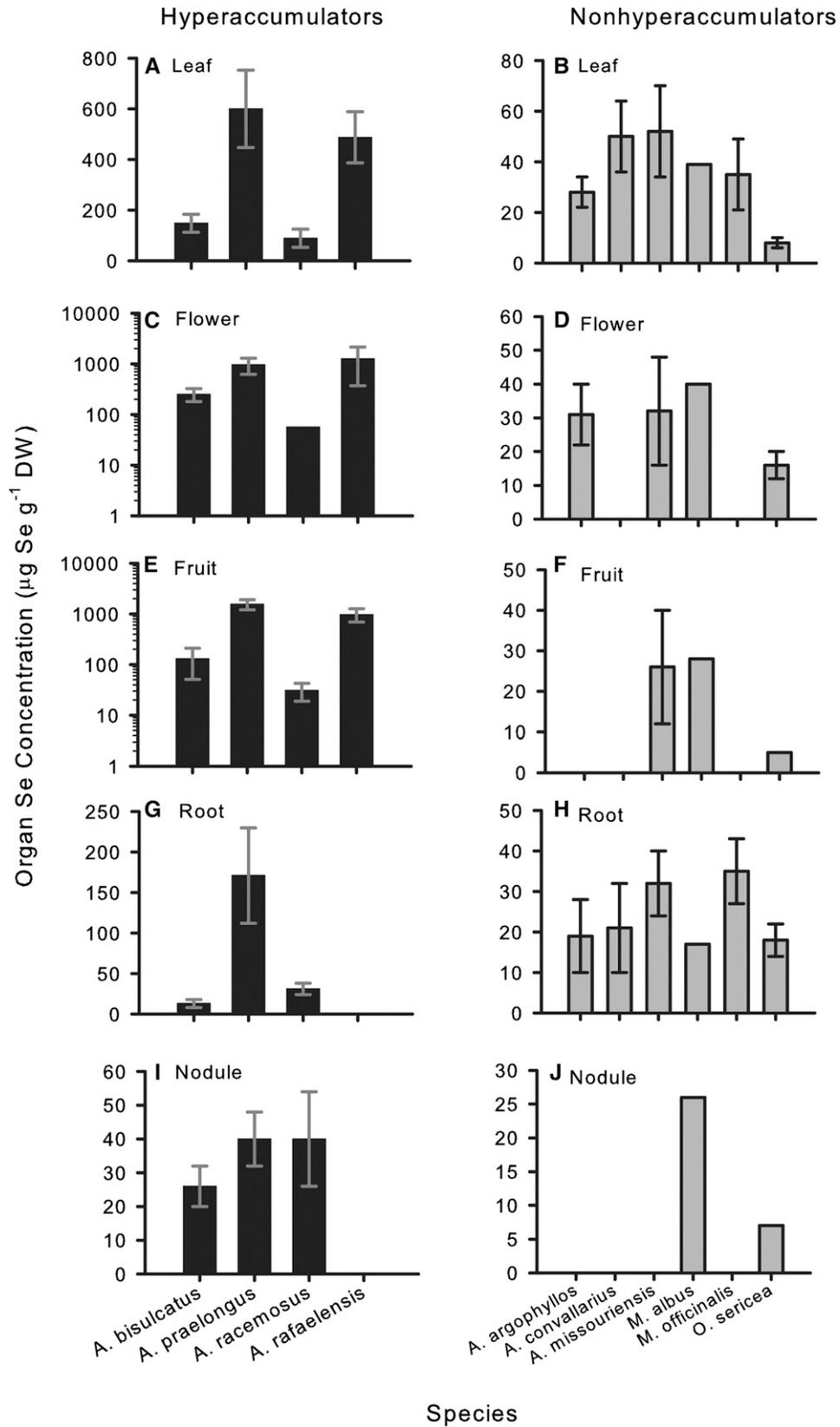


Fig. 2. Mean (±SE) [Se] in four Se-hyperaccumulator species (*Astragalus bisulcatus*, *A. praelongus*, *A. racemosus*, and *A. rafaensis*) as shown for (A) leaf, (C) flower, (E) fruit, (G) root, and (I) nodule, and (B) leaf, (D) flower, (F) fruit, (H) root, and (J) nodule of six nonhyperaccumulator legume species (*A. argophyllus*, *A. convallarius*, *A. missouriensis*, *Melilotus albus*, *M. officinalis*, and *Oxytropis sericea*).

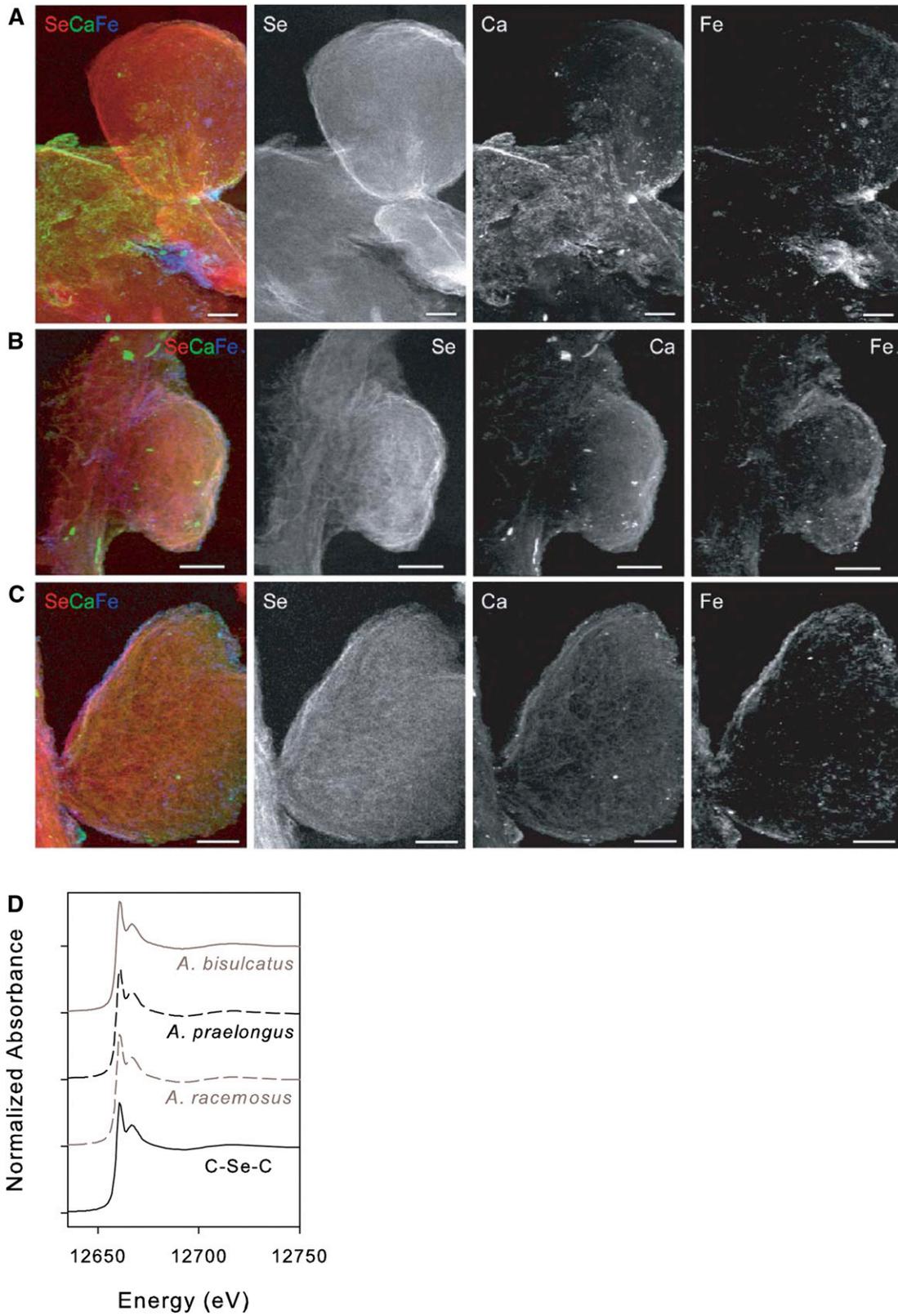


Fig. 3. Microfocussed x-ray fluorescence (μ XRF) maps derived from elemental distributions within (A) *Astragalus bisulcatus*, (B) *A. praelongus*, and (C) *A. racemosus* root nodules. The tricolor-coded maps show Se (in red), Ca (in green), and Fe (in blue) along with individual elemental maps (each shown in white). Microfocussed x-ray absorption near-edge structure (μ XANES) spectra from nodules from each of the *Astragalus* species along with the spectra of the standard organic Se, MeSeCys, containing carbon-Se-carbon bonds (D).

expected to have a lower value than nonhyperaccumulators. Statistical analyses were conducted with Systat v.12 (Systat Software, Chicago, Illinois, USA).

RESULTS

Nodulation occurrence in Se-hyperaccumulators—Nodulation of *Astragalus bisulcatus* and *A. racemosus* was confirmed in plants growing in the field. We observed nodulation in *A. praelongus* growing in its native habitat. Nodules were also observed in *A. crotalariae* and *A. preussii* inoculated with *A. bisulcatus* field soil under greenhouse conditions.

Organ [Se] from field collections—Ten legume species were collected from Colorado field sites (see Table 1) and analyzed for [Se] in leaves, flowers, fruits, roots, and nodules. Histograms show the number of plant samples within a range of [Se] for both Se-hyperaccumulators and nonhyperaccumulators (Fig. 1). Concentrations in hyperaccumulators and nonhyperaccumulators were variable, with nonhyperaccumulators having lower [Se] than hyperaccumulators. Except for hyperaccumulator flowers, both groups of plants have samples from each organ within the lowest ranges of [Se].

Astragalus missouriensis Nutt. had the lowest [Se] of the nonhyperaccumulators for leaf ($0 \mu\text{g Se g}^{-1} \text{DW}$), flower ($0.1 \mu\text{g Se g}^{-1} \text{DW}$), and fruit ($0 \mu\text{g Se g}^{-1} \text{DW}$). *A. argophylos* Nutt. had the lowest root and nodule [Se] at $0 \mu\text{g Se g}^{-1} \text{DW}$ for both organs. Nonhyperaccumulators did not surpass a [Se] of $100 \mu\text{g Se g}^{-1} \text{DW}$ in any organ, while every organ measured in hyperaccumulators surpassed that concentration. The highest leaf ($83 \mu\text{g Se g}^{-1} \text{DW}$) and fruit ($48 \mu\text{g Se g}^{-1} \text{DW}$) [Se] in nonhyperaccumulators were recorded for *A. missouriensis* on the seleniferous site in Fort Collins, Colorado (Table 3). *A. argophyllus* growing on a nonseleniferous site near Meeker, Colorado had the highest flower ($58 \mu\text{g Se g}^{-1} \text{DW}$) and root ($66 \mu\text{g Se g}^{-1} \text{DW}$) [Se] of the nonhyperaccumulators (Table 3). The maximum nodule [Se] found among all the nonhyperaccumulators was $29 \mu\text{g Se g}^{-1} \text{DW}$ in *Oxytropis sericea* growing at a seleniferous site near Fort Collins, Colorado (Table 3).

In contrast, *Astragalus praelongus* growing near Uravan, Colorado had the highest leaf ($2,925 \mu\text{g Se g}^{-1} \text{DW}$), flower ($2,999 \mu\text{g Se g}^{-1} \text{DW}$), fruit ($5,405 \mu\text{g Se g}^{-1} \text{DW}$), and root ($1,281 \mu\text{g Se g}^{-1} \text{DW}$) [Se] of all the Se-hyperaccumulators (Table 3). Maximum nodule [Se] in hyperaccumulators was in *A. racemosus* growing near Pueblo, Colorado, USA at $109 \mu\text{g Se g}^{-1} \text{DW}$ (Table 3). The lowest [Se] found in hyperaccumulators were $0 \mu\text{g Se g}^{-1} \text{DW}$ in some *A. racemosus* leaf, fruit, and root samples. Samples of *A. bisulcatus* had the lowest fruit ($55 \mu\text{g Se g}^{-1} \text{DW}$) and nodule ($6 \mu\text{g Se g}^{-1} \text{DW}$) [Se] of the hyperaccumulators.

Of the Se-hyperaccumulators, *Astragalus praelongus* had the highest average [Se] for leaves (Fig. 2A), fruits (Fig. 2E), and roots (Fig. 2G). *A. rafaensis* M. E. Jones had the highest average [Se] in flowers (Fig. 2C) and *A. racemosus* had the highest [Se] in nodules (Fig. 2I) of the hyperaccumulators. In the nonhyperaccumulators *A. missouriensis* had the highest average leaf [Se] (Fig. 2B); *Melilotus albus* Medik. had the highest average [Se] in flowers (Fig. 2D), fruit (Fig. 2F), and nodules (Fig. 2J); and *M. officinalis* (L.) Lam. had the highest average root [Se] (Fig. 2H).

Se localization and speciation in nodules—Selenium was distributed throughout root nodules in the three *Astragalus* hyperaccumulators, *A. bisulcatus* (Fig. 3A), *A. praelongus*

(Fig. 3B), and *A. racemosus* (Fig. 3C). Clear differences in Se compartmentalization in the developmentally different distal and proximal regions of the nodule were not apparent. The distribution of calcium (Ca) and iron (Fe) was more localized than Se, which was distributed more evenly in each nodule, but proximal or distal differences in Ca and Fe were not observed either. Molecular speciation by XANES showed that the majority of Se in the nodules of each plant species was C-Se-C (Fig. 3D; Table 4). Each root nodule also contained some selenite (SeO_3^{2-}). Both *A. bisulcatus* and *A. praelongus* contained some form of elemental Se (Se^0), but *A. racemosus* did not (Table 4). Nodules from *A. praelongus* contained selenocysteine, while nodules from *A. bisulcatus* and *A. racemosus* contained selenogluthathione.

Root and nodule [Se] under greenhouse and field conditions—Organ [Se] was significantly different between roots and nodules ($t = -3.090$; $\text{df} = 8$; $P = 0.013$) in the Se-hyperaccumulator *Astragalus bisulcatus* growing under greenhouse conditions (Fig. 4A). Nodules had a double the [Se] as roots in *A. bisulcatus* under greenhouse conditions. A similar trend (nonsignificant) where nodules had a higher [Se] than roots was observed in *A. bisulcatus* (Fig. 4B) and *A. racemosus* (Fig. 4D) growing under field conditions. In *A. praelongus* root [Se] was significantly higher ($t = 2.213$; $\text{df} = 23$; $P = 0.037$; +432%) than nodule [Se] (Fig. 4C).

Se effect on nodulation—Compared to nonhyperaccumulators, hyperaccumulators did not exhibit inhibition of nodulation characteristics (Fig. 5). Among the individual species, the nonhyperaccumulators *Astragalus shortianus* had the highest nodulation index, *A. convallarius* had the lowest, and all other species fell between these two (Fig. 5A). Additionally there was no difference in the nodulation index between the Se-hyperaccumulators and the nonhyperaccumulators as a group (Fig. 5B). The average weight of nodules was also determined. The Se-hyperaccumulators *A. praelongus* had the largest nodules and *A. bisulcatus* had the smallest nodules of all species with all other species falling between these two (Fig. 5C). No difference in nodule weight was found between the Se-hyperaccumulators and nonhyperaccumulators as a group (Fig. 5D).

Exposing *Astragalus* species to Se under greenhouse conditions did not alter the number of nodules produced in the Se-hyperaccumulator *A. bisulcatus* (Fig. 6A) or the nonhyperaccumulators *A. convallarius* (Fig. 6D) and *A. shortianus* (Fig. 6F). The addition of Se significantly increased nodule production in the hyperaccumulators *A. praelongus* ($F_{2,45} = 5.629$; $P = 0.007$; Fig. 6B) and *A. racemosus* ($F_{2,45} = 3.195$; $P = 0.050$; Fig. 6C). Only in the nonhyperaccumulator *A. drummondii* did Se addition significantly reduce plant nodule production ($F_{2,44} = 6.857$; $P = 0.003$; Fig. 6E). Across all three Se-hyperaccumulator species, adding Se in our greenhouse experiment significantly increased (+143%; $F_{2,140} = 3.409$; $P = 0.036$) the number of nodules formed by those plants (Fig. 6G). There was no significant effect of Se addition on nodule production when the three nonhyperaccumulators were analyzed as a group (Fig. 6H).

DISCUSSION

The results reported here do not support our hypothesis that Se hyperaccumulation inhibits nodulation in *Astragalus*. To our knowledge, we provide the first report of nodulation in three

TABLE 4. The percent of Se in each molecular species from nodules of three *Astragalus* hyperaccumulators as determined by Se K-edge x-ray absorption near edge spectroscopy (XANES).

	SeO ₃	C-Se-C	SeCys	SeGSH ₂	Se ⁰
<i>A. bisulcatus</i>	4	89	0	2	6
<i>A. praelongus</i>	2	70	22	0	6
<i>A. racemosus</i>	19	45	0	32	0

Astragalus Se-hyperaccumulators, *A. crotalariae*, *A. praelongus*, and *A. preussii*. All form nodules under greenhouse or field conditions. We also observed nodules on the hyperaccumulators *A. bisulcatus* and *A. racemosus* under greenhouse and field conditions, consistent with reports from Wilson and Chin (1947) and Lindblom et al. (2012). In the field we had the most success finding root nodules when the roots grew around rocks or between horizontal rocky layers, in locations where moisture was retained. A consistent pattern of nodulation in all hyperaccumulators examined indicates that symbiotic nodulation interactions are not diminished by a plant's ability to hyperaccumulate Se.

When we compared Se-hyperaccumulators to nonhyperaccumulators grown under greenhouse conditions we found no evidence that Se treatment inhibited the formation of root nodule symbiosis in hyperaccumulators. These findings are similar to the report of a successful symbiotic interaction in the metal-

lophyte *Lotus purshianus* growing on an abandoned copper mine (Wu and Lin, 1990), except here we find that tolerance is widespread in numerous species of *Astragalus*. Our findings are in contrast to reduced symbiotic dependence found in some metal hyperaccumulators, where strong Ni-hyperaccumulators native to New Caledonia had reduced mycorrhizal colonization compared to species that are moderate or weak accumulators of Ni (Amir et al., 2007). When we added Se to hyperaccumulators under greenhouse conditions the number of nodules per plant increased with increasing Se doses. This was not the case in the nonhyperaccumulators where there was no significant effect of adding Se. These effects for nonhyperaccumulator legumes agree with the earlier report that Se had no effect on nodule number in *Melilotus indicus* (L.) All., although Se additions did reduce mean nodule dry weight in that study (Wu et al., 1994). However, in our study the Se-hyperaccumulators made 143% more nodules when they were given up to 100 μ M Se, indicating that the belowground symbiosis in hyperaccumulators is positively linked in some way to Se.

We hypothesize that nodulation could be linked to Se hyperaccumulation where increased nodule production under Se treatment increases plant N content, which could promote the storage of selenoamino acids in Se-hyperaccumulators. This is feasible to imagine because *Astragalus bisulcatus* stores up to 99% of Se in young leaves as the selenoamino acid methylselenocysteine (Sors et al., 2005). Investigations of the tolerance of

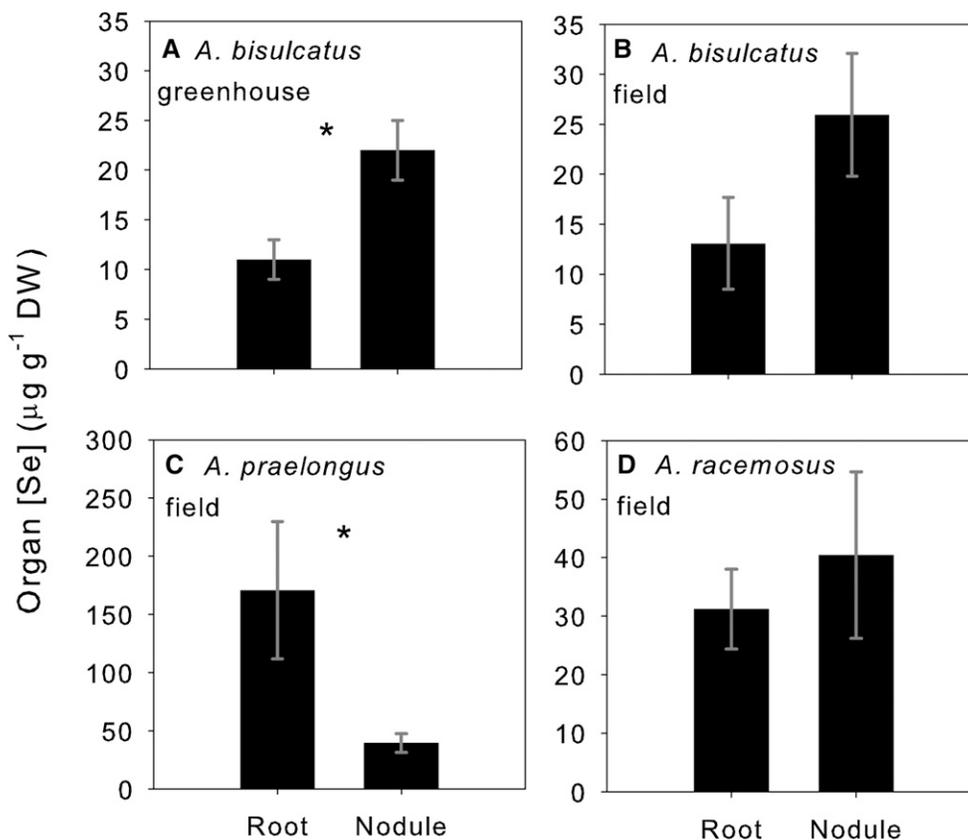


Fig. 4. Mean [Se] in roots and nodules (\pm SE) of *Astragalus* Se-hyperaccumulators. (A) *A. bisulcatus* under greenhouse conditions, (B) *A. bisulcatus* under field conditions, (C) *A. praelongus* under field conditions, and (D) *A. racemosus* under field conditions. The asterisk (*) indicates a significant difference between belowground organs.

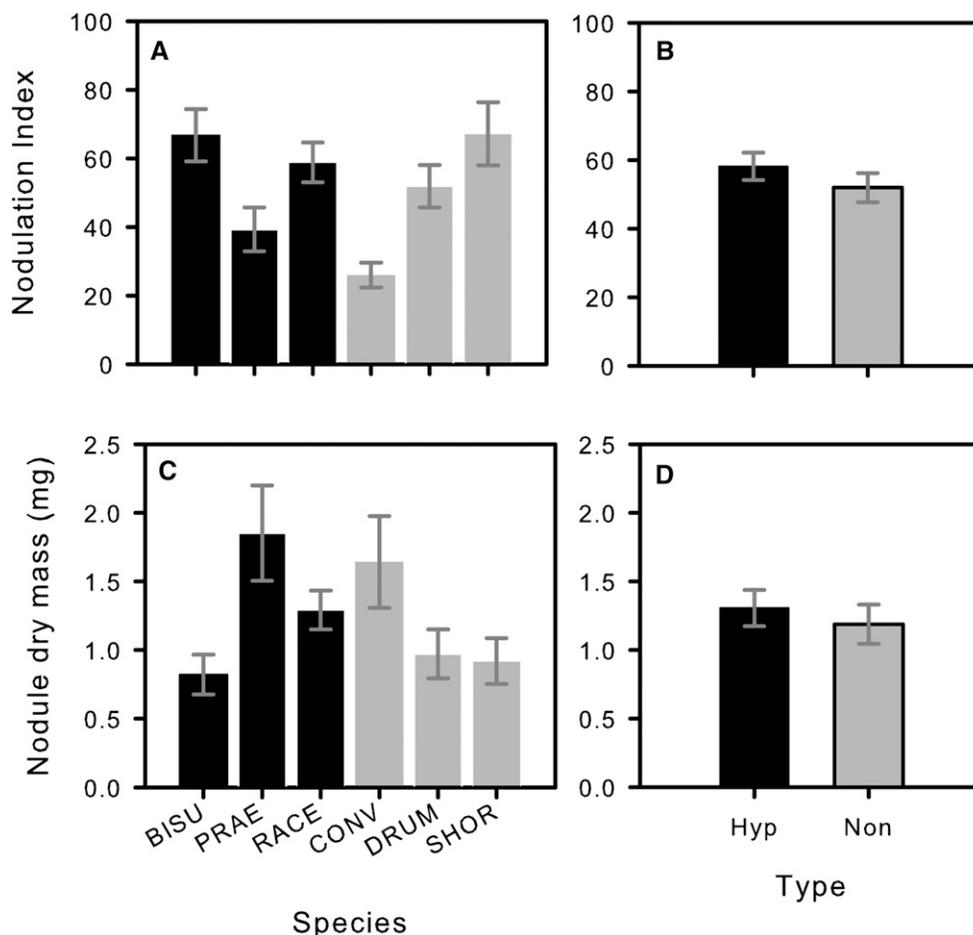


Fig. 5. Nodulation traits measured in three *Astragalus* Se-hyperaccumulators (black bars) [*A. bisulcatus* = BISU; *A. praelongus* = PRAE; and *A. racemosus* = RACE] compared to three *Astragalus* nonhyperaccumulators (gray bars) [*A. convallarius* = CONV; *A. drummondii* = DRUM; and *A. shortianus* = SHOR]. Nodulation index (determined by the number of nodules produced per gram of shoot dry weight) (A) for each individual species and (B) for each Se-accumulation type. Dry mass per nodule (mg) for (C) each individual species and (D) the two Se-accumulation types.

this symbiosis may lead to a better understanding of the evolution of the plant species as well as the coevolutionary forces at work in these interactions.

The maximum organ [Se] for each species growing under field conditions was tested to provide a reference to amount of Se in the leaf ($1,000 \mu\text{g Se g}^{-1}$ DW) by which Se-hyperaccumulators are defined in the literature (Brooks, 1998). We did not find nodule [Se] in hyperaccumulators at that level, but all other organs surpassed the $1,000 \mu\text{g Se g}^{-1}$ DW threshold. Nodule [Se] did surpass the $100 \mu\text{g Se g}^{-1}$ DW level in the Se-hyperaccumulator *Astragalus racemosus*. Organic Se forms are often the major Se constituents stored in organs in *Astragalus* hyperaccumulators (Valdez Barillas et al., 2012). *A. bisulcatus* roots, nodules, and young leaves store larger quantities of organic Se than other Se forms (Pickering et al., 2003; Freeman et al., 2006; Lindblom et al., 2012; Valdez Barillas et al., 2012). Leaves, nodules, and roots of *A. racemosus* have also been shown to contain large fractions of organic Se (Lindblom et al., 2012). Inside the root nodules of all three *Astragalus* Se-hyperaccumulators we found that the majority of Se was stored in organic forms, mainly as C-Se-C compounds. This result from nodules of three different Se-hyperaccumulators growing under field and greenhouse conditions matches the findings reported

for field-collected nodules of *A. bisulcatus*, where a root nodule collected from the field contained a majority of Se in the C-Se-C form (Valdez Barillas et al., 2012). Neither our report of three Se-hyperaccumulator species, nor the Valdez Barillas et al. (2012) report on *A. bisulcatus* indicates that Se is localized differently between the young, distal end of nodules and the older, proximal region.

The leguminous nonhyperaccumulators that we investigated never breached the $100 \mu\text{g Se g}^{-1}$ DW level in any organ, even when co-occurring with hyperaccumulators on seleniferous soil. Our finding is similar to previous reports of nonhyperaccumulator species growing on seleniferous sites (Shrift 1969, Galeas et al., 2007). Examining the nodule [Se] of nonhyperaccumulators is new, but like the other organs, [Se] was low. The maximum nodule [Se] found in nonhyperaccumulators was $29 \mu\text{g Se g}^{-1}$ DW. In fact, an attempt to analyze the nodule of the nonhyperaccumulator *Astragalus drummondii* by μXAS was unsuccessful because of the low Se signal in the sample analyzed.

While mostly investigated aboveground, the protective effect of trace-element hyperaccumulation may also occur belowground. To our knowledge the elemental defense hypothesis (Boyd, 2007) has never been demonstrated in belowground organs, although in some species belowground organs do satisfy

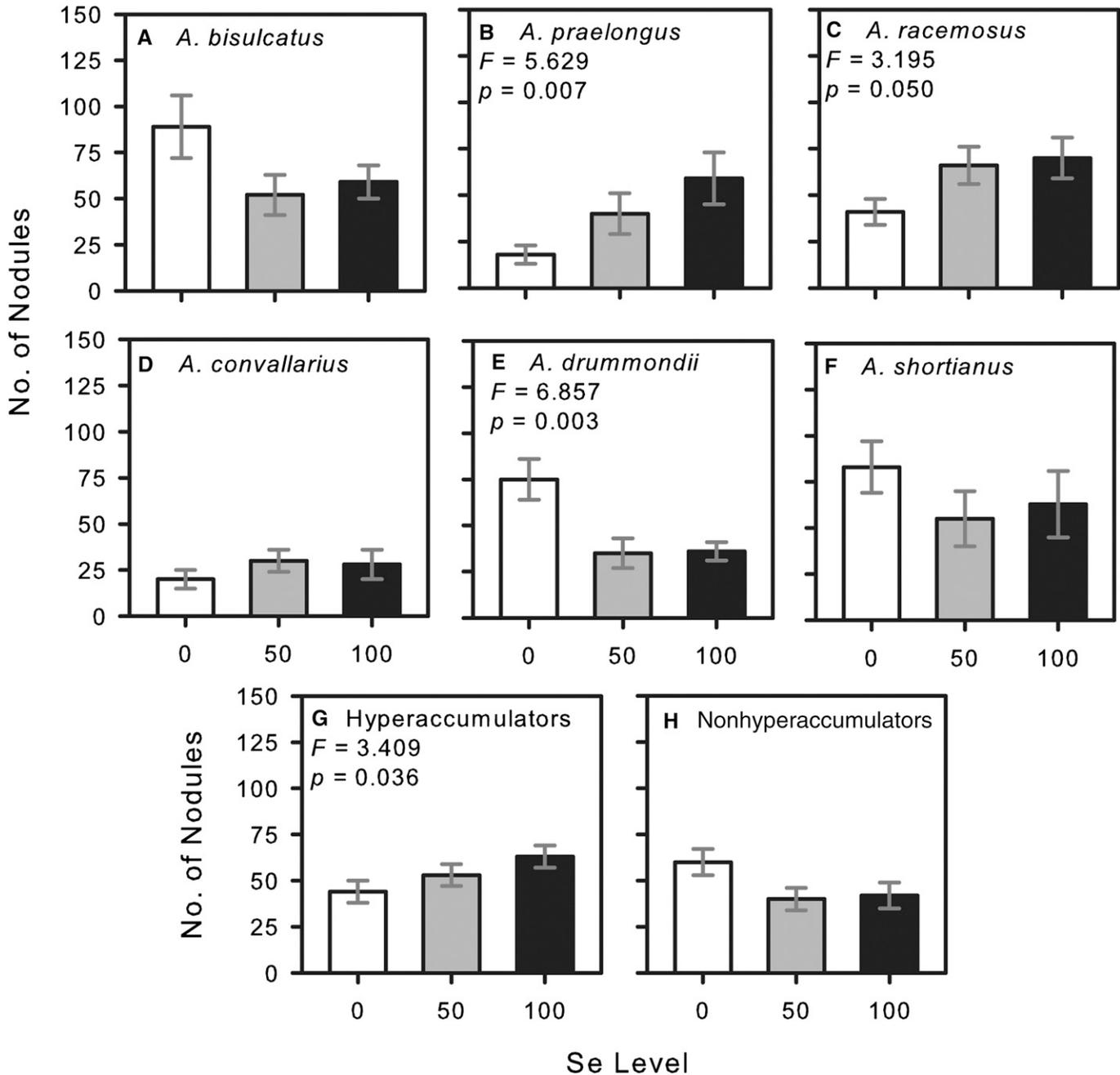


Fig. 6. The effect of Se addition (0, 50, or 100 µM) on the number of root nodules produced per plant growing under greenhouse conditions in the Se-hyperaccumulators (A) *Astragalus bisulcatus*, (B) *A. praelongus*, and (C) *A. racemosus*, and the nonhyperaccumulators (D) *A. convallarius*, (E) *A. drummondii*, and (F) *A. shortianus*. Also shown are (G) the effect of Se addition on nodulation in hyperaccumulators as a group and (H) nonhyperaccumulators as a group. Significant responses are indicated by listing *p*-values.

the concentration criteria used to classify plants as hyperaccumulators (Amir et al., 2007; Barzanti et al., 2007; Galeas et al., 2007; Wu et al., 2007; Barillas et al., 2011; this study). Our finding that nodule [Se] was higher than root [Se] under greenhouse conditions in *Astragalus bisulcatus* suggests a role for Se in nodule defense in this species. Nodules, which are expensive organs for the plant to construct and maintain, may be protected from herbivory by Se. It is known that larval weevils of *Sitona lepidus* (Coleoptera: Curculinidae) feed on root nodules of clover (Johnson et al., 2004), but to our knowledge no studies have

investigated root nodule herbivory in *Astragalus*. Our study was not designed to test the elemental defense hypothesis, but these greenhouse results suggest new aspects to investigate. In contrast with our greenhouse findings, when we looked at root and nodule [Se] in other *Astragalus* Se-hyperaccumulators growing under field conditions, the opposite result was also detected where root [Se] was higher than nodule [Se]. A more intensive study of belowground Se translocation must be conducted because of the known variation of Se allocation during the growing season (Galeas et al., 2007).

Selenium cycles through the plant and is proposed to move from the roots to the new leaves in the spring, from the mature leaves into the flowers and fruits, and back to the roots during dormancy (Galeas et al., 2007). This cycle does not account for root nodules. The data we collected here were from actively growing plants when Se is translocated from roots to above-ground organs. We expect there is better potential to detect differences in belowground organs when Se moves back belowground. Perennial legumes are expected to have perennial nodules (Gurusamy et al., 2000), so Se cycling within plants could manifest in nodules as well. If nodules are inactive during the overwintering process, Se may not affect nodule physiology, but may rather affect their susceptibility to herbivory. Even if nodule [Se] is elevated in hyperaccumulators, bacteria inside nodules are separated from the plant cell by the peribacteroid membrane (Brewin, 1991). Therefore, nodule [Se] may be isolated from the bacteria. However, free-living rhizobia must cross the Se-enriched rhizosphere soil from Se-hyperaccumulators to infect the host root. Even though *Astragalus* Se-hyperaccumulators do not show evidence of reduced reliance on symbiotic root nodule interactions, the coevolutionary effects of Se-hyperaccumulation on bacterial symbionts remain to be determined.

Astragalus nodule [Se] may be related to the promiscuity of rhizobia that associate with *Astragalus* hyperaccumulators and nonhyperaccumulators. We did not address this, but different nodule [Se] between Se-hyperaccumulators and nonhyperaccumulators could act as a selective force for rhizobial symbionts. Rhizosphere fungal communities from seleniferous soils have been shown to have increased Se tolerance and some may be considered habitat specialists that associate with Se-hyperaccumulators (Wangeline et al., 2011). Tolerance to inorganic Se forms at levels that well-exceed the nodule [Se] in all our specimens have been observed in some rhizobia isolates in vitro (Kinkle et al., 1994). The maximum nodule [Se] found under field conditions in this study was 109 $\mu\text{g Se g}^{-1}$ DW, which is much lower than the 200 mM Se (IV) or 400 mM Se (VI) minimum inhibitory concentrations (MIC) determined by Kinkle et al. (1994) for *Rhizobium leguminosarum* bv. *viciae*. Since some rhizobia have a high Se tolerance, the same bacteria that infect nonhyperaccumulators have the potential to infect Se-hyperaccumulators as well. If so, co-occurring *Astragalus* species would not have segregated symbionts, but rather each host species promotes the growth of the same bacterial symbiont, thereby enhancing the rhizobial population within sites where the two *Astragalus* groups co-occur. Whether or not Se serves as a selective force, microsymbiont identities of many perennial milkvetch species in North America remains to be investigated by molecular methods.

Just like their nonhyperaccumulator congeners, *Astragalus* Se-hyperaccumulators form root nodule symbioses. The hyperaccumulators differed from their nonhyperaccumulator congeners in the fact that their symbiotic relationship is related to Se treatment and accumulation, while these effects did not occur in nonhyperaccumulators. We hypothesize that the increased number of nodules in Se-hyperaccumulators treated with increased Se levels may result in the symbiotic interaction helping plants acquire more N, which in turn helps the plant to store more Se as selenoamino acids in their shoots. These interactions present opportunities for further studies of evolutionary relationships. We found that the symbiotic interaction is not inhibited by the plant's ability to hyperaccumulate Se. Because of the Se translocation cycle within perennial *Astragalus*

Se-hyperaccumulator species, nodule [Se], symbiont exposure, and the consequences of those levels for segregation of symbionts between co-occurring plant congeners remain to be determined in a more conclusive way.

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